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Correlates of serum hepcidin levels and its association with cardiovascular disease in an elderly general population

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Abstract

Background: The expression of the key iron regulatory hormone hepcidin is regulated by iron availability, inflammation, hormones, hypoxia, and anaemia. Increased serum concentrations of hepcidin have recently been linked to atherosclerosis. We studied demographic, haematologic, biochemical, and dietary correlates of serum hepcidin levels and its associations with incident cardiovascular disease and with carotid atherosclerosis.
Methods: Serum hepcidin concentrations were measured by tandem mass spectrometry in samples taken in 2000 from 675 infection-free participants of the prospective population-based Bruneck study (age, mean±standard deviation, 66.0±10.2; 48.1% male). Blood parameters were measured by standard methods. Dietary intakes of iron and alcohol were surveyed with a food frequency questionnaire. Carotid atherosclerosis (365 cases) was assessed by ultrasound and subjects were observed for incident stroke, myocardial infarction, or sudden cardiac death (91 events) until 2010.
Results: Median (interquartile range) hepcidin levels were 2.27 nM (0.86, 4.15). Most hepcidin correlates were in line with hepcidin as an indicator of iron stores. Independently of ferritin, hepcidin was related directly to physical activity (p=0.024) and fibrinogen (p<0.0001), and inversely to alcohol intake (p=0.006), haemoglobin (p=0.027), and γ-glutamyltransferase (p<0.0001). Hepcidin and hepcidin-to-ferritin ratio were not associated with prevalent carotid atherosclerosis (p=0.43 and p=0.79) or with incident cardiovascular disease (p=0.62 and p=0.33).
Conclusions: In this random sample of the general community, fibrinogen and γ-glutamyltransferase were the most significant hepcidin correlates independent of iron stores, and hepcidin was related to neither atherosclerosis nor cardiovascular disease.

Keywords: atherosclerosis; biomarkers; cardiovascular disease; hepcidin; iron metabolism.

Introduction

The liver-derived peptide hormone hepcidin is the master regulator of systemic iron homeostasis [1, 2]. Hepcidin expression is induced mainly in hepatocytes by iron loading, inflammatory signals, and endoplasmic reticulum stress whereas iron deficiency, hypoxia, anaemia, and several hormones reduce the formation of this peptide [1–7]. Hepcidin acts upon binding to the only known cellular iron export protein ferroportin, resulting in its internalisation, degradation and thus blockage of cellular iron egress [8]. As a consequence, hepcidin reduces duodenal iron absorption and iron release from hepatocytes and macrophages, thereby lowering systemic iron levels and shifting iron from plasma to iron stores [2, 9].

Iron loading has been associated with an increased risk of atherosclerosis [10–12], and thus recent studies have evaluated a potential association of hepcidin with atherosclerosis and the putative utility of hepcidin

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as a diagnostic or predictive marker in cardiovascular disease (CVD) [10, 13–16]. Hepcidin may predispose to atherosclerotic lesions and CVD by enhancing iron retention in plaque macrophages, thereby increasing iron catalysed oxidative stress and lipid oxidation, and promoting foam cell formation and plaque instability [10], which is further aggravated by hepcidin-mediated inhibition of macrophage cholesterol efflux [13]. However, the cause effective roles of iron and hepcidin for the pathogenesis of atherosclerosis are still debated [11, 12, 16]. Experimental studies revealed conflicting results [13–15], and epidemiologic reports have mostly, but not exclusively [17], been restricted to selected patient populations [18–20].

This work aimed to comprehensively analyse the associations between hepcidin serum concentration and its correlates and to scrutinise the associations of hepcidin with carotid atherosclerosis and, for the first time, with its clinical sequels stroke, myocardial infarction and sudden cardiac death.

Materials and methods

An unabridged description of the study population and materials and methods is available in the online-only Supplemental Data that accompanies the article at http://www.degruyter.com/view/j/cclm.2016.54.issue-1/cclm-2015-0068/cclm-2015-0068.xml?format=INT

Study population and data collection

The Bruneck Study is a prospective, population-based survey on the epidemiology and pathogenesis of atherosclerosis and CVD [21–23]. At baseline in 1990 the study population comprised an age- and sex-stratified random sample of all inhabitants of Bruneck (125 men and 125 women from each of the fifth through eighth decades of age, all of Western European descent; 93.4% participated). In 2000, 702 subjects participated in the second quinquennial re-examination and serum samples for hepcidin measurement were available in 694 individuals. During follow-up from 2000 to 2010, detailed information about fatal and non-fatal new-onset CVD was carefully collected for all of these 694 subjects (follow-up rate, 100%). The study protocol was approved by the Ethics Committees of Bolzano and Verona and conforms to the Declaration of Helsinki. All study subjects provided written informed consent. Risk factors were assessed by means of validated standard procedures as described previously [21–24]. Blood samples were drawn in the morning between 7 and 9 AM after an overnight fast and 12 h of abstinence from smoking. In subjects with acute infection, blood sampling was delayed for at least 6 weeks, i.e. until at least 4–5 weeks after recovery from the respective infectious disease. Serum samples were divided into aliquots and immediately stored at −80 °C.

Hepcidin measurement

Hepcidin was quantified by tandem mass spectrometry using an adaptation of a published method [25] as detailed in the Supplemental Data.

Definition of clinical variables

Study participants underwent an extensive examination and completed a standardised questionnaire on medical history and lifestyle factors. Body mass index (BMI) was calculated as weight in kilograms over height in meters squared. Total and high-density lipoprotein cholesterol were measured by standard procedures. Glomerular filtration rate was estimated based on serum creatinine according to the Modification of Diet in Renal Disease (MDRD) formula. Transferrin saturation was derived from iron and transferrin concentrations, which were measured directly. Ferritin index was calculated as soluble transferrin receptor (mg/L) divided by $\log_{10}$ ferritin (µg/L).

Anaemia was defined as haemoglobin <120 g/L in women and <130 g/L in men. Aetiologies of anaemia were classified according to common criteria as detailed in the Supplemental Data.

Ascertainment of physical activity and of dietary intakes

We ascertained physical activity by the Baecke questionnaire, rated activity intensities according to the compendium of physical activities [26], and calculated average metabolic equivalent hours per week to quantify long-term physical activity. We assessed food intakes by a standardised dietician-supervised food-frequency questionnaire (FFQ).

Definition of atherosclerosis

Carotid artery ultrasound scans were recorded in 2000 for all subjects in a supine position using a duplex ultrasound system with a 10-MHz transducer [21, 27, 28]. Four segments of the right and left carotid artery were examined in multiple planes: proximal common carotid artery (15–30 mm proximal to the carotid bulb), distal common carotid artery (<15 mm proximal to the carotid bulb), proximal internal carotid artery (carotid bulb and the initial 10 mm of the vessel), distal internal carotid artery (>10 mm above the flow divider). A plaque was defined as an echo structure protruding into the lumen with focal broadening of the vessel wall of at least 0.5 mm relative to adjacent segments.

Definition of the clinical endpoint

The composite CVD endpoint comprised incident ischaemic stroke, fatal and non-fatal myocardial infarction, and sudden cardiac death (n=91). Presence of myocardial infarction was assessed by World Health Organization criteria (definite disease status) [29] while stroke was classified according to the criteria of the National Survey of Stroke [30].

Statistical analysis

Data are presented as mean±standard deviation, median (first quartile, third quartile), or count (percentage). p-Values were calculated by t-test, Wilcoxon-Mann-Whitney test, χ^2-test or Fisher's exact test as appropriate. The non-linear dependency of hepcidin concentration on age is shown using smooth 0.10, 0.25, 0.50, 0.75, and 0.90 quantiles fitted by quantile regression [31].

Associations between hepcidin and other variables were examined by ordinary least squares regression. The dependent variable hepcidin and the independent variables ferritin, fibrinogen, hs-CRP, alanine aminotransferase, and γ-glutamyltransferase (γ-GT) were $\log_2$-transformed towards normality for these inferential analyses, and regression coefficients were back-transformed.

Associations of hepcidin and derived variables with atherosclerosis were studied using logistic regression, and associations with CVD using Cox regression. The proportional hazards assumption was tested by computing the correlation between Schoenfeld residuals and follow-up time and was not refuted. These results are presented with adjustment for age, sex, C-reactive protein, anaemia, and the Framingham risk score variables age, sex, diabetes mellitus, current smoking, systolic blood pressure, and total and high-density lipoprotein cholesterol, which were summarised utilising full-cohort disease risk scores. P for trend was obtained by using hepcidin, hepcidin-to-ferritin ratio, or hepcidin-to-transferrin saturation ratio as log-transformed continuous predictor.

We used bootstrap aggregated backwards stepwise linear regression (1000 repetitions) based on Bayesian Information Criterion minimisation to identify independent predictors of hepcidin concentration from those univariably significant in Table 2 in addition to age and sex.

More detail on statistical methods employed is given in the Supplemental Data. An α level of 0.05 is used throughout and all p-values are two-sided. Analyses were conducted with R 3.1.0.

Results

Hepcidin could be measured in 675 of 694 participating subjects (97.3%), whose baseline characteristics are shown in Table 1. Levels and distribution of serum hepcidin concentration by age and sex are shown in Figure 1. In women, of which 95.7% had undergone menopause, hepcidin levels on average increased by 15% per year from ages 50 to 60 (95% confidence interval, 5–25) and decreased by 3% (1–5) per year from ages 60 to 90. Average hepcidin levels in men did not change with age (p=0.32) and were approximately 51% (23–84) higher than in women independently of age. Conditional on serum ferritin men had 27% (14–38) lower average hepcidin levels. The relationship between hepcidin and ferritin in subjects with anaemia is shown in Figure 2. Compared to a median hepcidin concentration of 2.30 nmol/L (6.42 μg/L) in subjects without anaemia (n=651), median hepcidin concentrations in anaemic subjects by aetiology of anaemia were as follows: iron deficiency anaemia, 0.10 nmol/L (0.28 μg/L; n=9); vitamin B12 or folate deficiency anaemia, 4.31 nmol/L (12.0 μg/L; n=3); anaemia of inflammation, 3.88 nmol/L (10.8 μg/L; n=3); anaemia of multiple aetiologies, 2.20 nmol/L (6.1 μg/L; n=3).

Results on the relationships between hepcidin and iron, haematologic, inflammatory, and dietary variables in all study subjects and by sex are given in Table 2, and conditional on body iron stores assessed as ferritin in Table 3. Associations of hepcidin with iron parameters were generally highly significant (most p-values <0.001) and similar between sexes.

There were direct relationships of hepcidin with hs-CRP and with fibrinogen, but only that with fibrinogen was robust to adjustment for ferritin. We also examined the simultaneous associations of these inflammation markers with hepcidin upon adjustment for age, sex, and body mass index. Fibrinogen (p=0.008) but not CRP (p=0.24) was independently and significantly associated with circulating hepcidin.

γ-GT was highly significantly negatively related to hepcidin under adjustment for ferritin but not without such adjustment. Other kidney and liver parameters displayed non- or only weakly significant associations with hepcidin, which were poorly consistent under varying multivariable adjustment and between sexes.

Dietary intakes of alcohol showed marked sex differences in their association with hepcidin concentration. In women, alcohol intake was negatively associated with hepcidin levels even after ferritin adjustment, whereas a positive association between these two variables became evident in men, and this association ceased with adjustment for ferritin.

Statistically selected independent predictors of hepcidin concentration are presented in Table 4. There were no interactions by sex and they are therefore only presented in the sexes combined.

We then studied the associations of hepcidin, of hepcidin-to-ferritin ratio, and of hepcidin-to-TfS ratio levels with the prevalence of ultrasound determined carotid atherosclerosis (Online Table 1). We found no significant associations between any of these variables and the presence of carotid plaque in men or women. For sensitivity purposes we also examined effects in sub groups of blood donors and non-donors, alcohol drinkers (>1 g/day) and non-drinkers, subjects with increased (>28.6 nmol/L, >3 mg/L) and with undetectable or low CRP (≤28.6 nmol/L), non-anaemic subjects, subjects not taking statins, and subjects without prior CVD, which yielded no significant results. We then evaluated a possible association between the same variables and the

Table 1: Baseline characteristics of the study population.

	All subjects	Men	Women	$p_{Sex\ difference}$
No. of subjects	675	325	350	
Age, years	66.0±10.2	65.3±10.0	66.6±10.4	NS
Demographic and lifestyle variables				
Body mass index, kg/m^2	25.4±4.0	25.4±3.5	25.5±4.5	NS
Body mass index >30 kg/m^2, n (%)	88 (13.3)	33 (10.4)	55 (15.9)	0.048
Physical activity, MET-h/week	50.8±39.4	58.3±42.7	43.7±34.7	<0.001
Blood donation, n (%)	112 (16.7)	94 (29.3)	18 (5.2)	<0.001
Hormone replacement therapy, n (%)			71 (20.3)	
Postmenopausal, n (%)			331 (95.7)	
Prior CVD, n (%)	44 (6.5)	27 (8.3)	17 (4.9)	NS
Iron variables				
Hepcidin, nmol/L	2.27 (0.86, 4.15)	2.54 (1.13, 4.85)	1.98 (0.62, 3.72)	<0.001
Ferritin, pmol/L	181.8 (96.6, 329.2)	266.5 (135.9, 465.4)	133.0 (77.1, 222.2)	<0.001
Hepcidin-to-ferritin ratio, mol/mol	11.0 (5.5, 18.6)	9.3 (4.5, 14.6)	13.6 (6.6, 21.2)	<0.001
Iron, µmol/L	18.7±6.5	19.7±7.0	17.7±5.8	<0.001
Serum transferrin, µmol/L	27.8±4.6	27.3±4.7	28.2±4.5	0.009
Transferrin saturation, %	31.7 (25.1, 40.6)	32.8 (26.6, 43.8)	30.8 (24.3, 37.9)	<0.001
Hepcidin-to-TfS ratio, pmol/L/%	65.4 (92.7)	73.3 (98.3)	61.5 (88.5)	0.007
Soluble transferrin receptor, mg/L	1.30 (1.16, 1.48)	1.26 (1.13, 1.43)	1.33 (1.18, 1.53)	<0.001
Ferritin index	0.69 (0.57, 0.84)	0.62 (0.51, 0.75)	0.76 (0.63, 0.94)	<0.001
Red blood cell variables				
Haemoglobin, g/L	144.3±12.1	150.7±11.0	138.3±9.8	<0.001
Haematocrit, %	42.4±3.5	44.2±3.2	40.7±2.9	<0.001
Erythrocyte count, ×10^{12}/L	4.60±0.42	4.75±0.43	4.46±0.36	<0.001
MCV, fL	92.2±5.3	93.2±5.7	91.2±4.8	<0.001
MCH, pg/cell	31.4±1.9	31.8±2.1	31.0±1.7	<0.001
MCHC, g/L	341±5	341±6	340±5	0.001
Red cell distribution width, %	13.0 (12.6, 13.5)	13.0 (12.6, 13.5)	13.0 (12.6, 13.5)	NS
Anaemia[a], n (%)	24 (3.6)	12 (3.7)	12 (3.4)	NS
Inflammation markers				
Fibrinogen, g/L	2.89±0.59	2.81±0.65	2.97±0.54	<0.001
hs-CRP, nmol/L	17.3 (8.8, 38.3)	16.3 (8.0, 36.3)	18.6 (9.3, 39.7)	NS
hs-CRP >28.6 nmol/L, n (%)	222 (32.9)	97 (29.8)	125 (35.7)	NS
Liver and kidney parameters				
eGFR-MDRD, mL/min/1.73 m^2	81.9±14.6	86.5±15.3	77.6±12.4	<0.001
eGFR-MDRD <60 mL/min/1.73 m^2, n (%)	45 (6.7)	18 (5.5)	27 (7.7)	NS
Alanine aminotransferase, µkat/L	0.35 (0.28, 0.48)	0.40 (0.30, 0.57)	0.33 (0.27, 0.42)	<0.001
Alanine aminotransferase >0.83 µkat/L, n (%)	45 (6.7)	32 (9.8)	13 (3.7)	0.002
γ-Glutamyltransferase, µkat/L	0.42 (0.27, 0.72)	0.52 (0.35, 0.90)	0.33 (0.23, 0.48)	<0.001
Dietary variables				
Iron intake, mg/day	13.7 (12.3, 15.3)	13.2 (11.9, 14.6)	14.2 (12.7, 16.4)	<0.001
Haeme iron intake, mg/day	0.80 (0.58, 1.08)	0.88 (0.62, 1.17)	0.76 (0.56, 0.98)	<0.001
Non-haeme iron intake, mg/day	12.7 (11.4, 14.3)	12.3 (10.8, 13.6)	13.2 (12.0, 14.9)	<0.001
Intake of iron supplements, n (%)	15 (2.2)	1 (0.3)	14 (4.0)	0.001
Alcohol intake, g/day	13.6 (4.4, 30.7)	28.7 (11.6, 51.9)	8.0 (2.4, 15.3)	<0.001
Alcohol intake >1 g/day, n (%)	504 (75.1)	301 (92.9)	203 (58.5)	<0.001

Values are given as mean±standard deviation, median (first quartile, third quartile), or count (percentage). CVD, cardiovascular disease; eGFR-MDRD, glomerular filtration rate estimated by the Modification of Diet in Renal Disease formula; hs-CRP, high-sensitivity C-reactive protein; MET-h/week, metabolic equivalent hours per week; MCH, mean cell haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NS, not significant. [a]Anaemia was defined as haemoglobin <120 g/L in women or <130 g/L in men. p-Values were calculated by t-test, Wilcoxon-Mann-Whitney test, χ^2-test or Fisher's exact test as appropriate. To convert serum hepcidin from nmol/L to ng/mL, multiply by 2.789. To convert ferritin from pmol/L to ng/mL, multiply by 0.445. To convert iron from µmol/L to µg/dL, multiply by 5.587. To convert serum transferrin from µmol/L to mg/dL, multiply by 8.130. To convert hs-CRP from nmol/L to mg/L, multiply by 1.908. To convert alanine aminotransferase or γ-glutamyltransferase from µkat/L to U/L, multiply by 59.880.

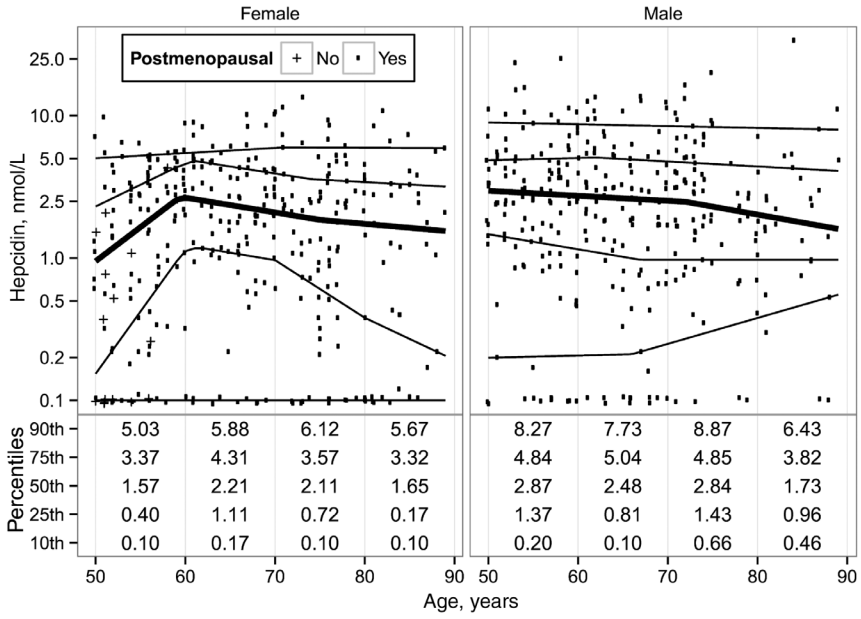

Figure 1: Serum concentrations of hepcidin by age and sex.
Upper panel: Points are jittered horizontally and points at the lower limit of detection also vertically to alleviate overplotting. Median, first and third quartiles, and 10th and 90th percentiles are shown as smooth quantiles. Lower panel: The same quantiles are given in decades of age (50–59, 60–69, 70–79, 80–89 years).

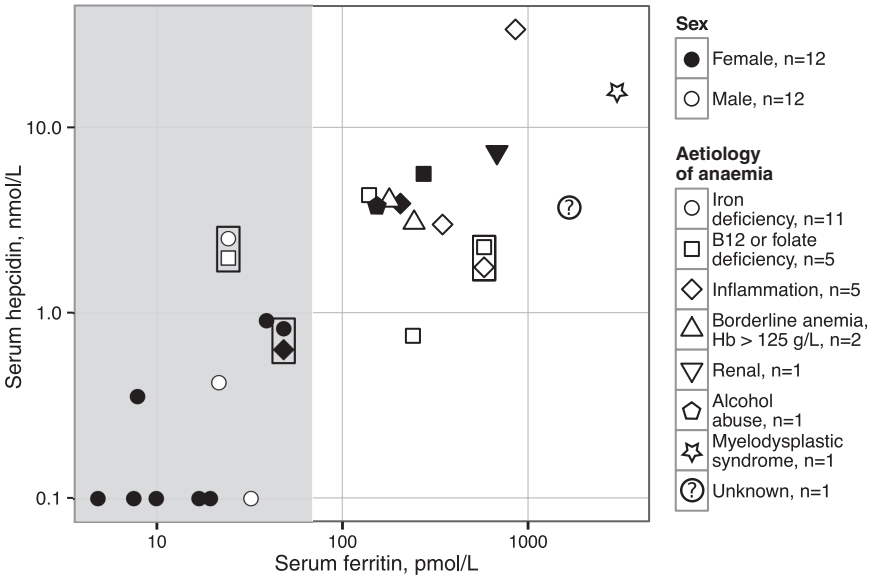

Figure 2: Joint distribution of hepcidin and ferritin in anaemic subjects by aetiology of anaemia and sex.
Symbols representing multiple aetiologies in the same subject are bordered by black rectangles. The grey shaded area marks iron deficiency as defined by ferritin <67.4 pmol/L.

emergence of composite CVD, defined as incident ischaemic stroke, fatal or non-fatal MI, or sudden cardiac death between 2000 and 2010 (Table 5). Although CVD events occurred in a total of 91 subjects, we did not detect any associations with CVD risk, irrespective of sex and also when examining the abovementioned sub groups.

Discussion

Herein we investigated hepcidin serum concentrations in a population-based study and examined its associations with laboratory and environmental factors, along with an analysis of its associations with atherosclerosis and

Table 2: Relationships of hepcidin serum concentration with demographic, blood, and dietary variables (95% confidence intervals) under adjustment for age and sex.

	All subjects	Men	Women	$p_{Interaction}$
Demographic and lifestyle variables				
Body mass index, kg/m²	2.7 (0.1, 5.3)[a]	2.5 (−1.7, 6.9)	2.7 (−0.4, 6.0)	NS
Physical activity, MET-h/week	0.19 (−0.08, 0.46)	0.41 (0.07, 0.75)[a]	−0.14 (−0.55, 0.28)	0.043
Postmenopausal			391 (140, 908)[c]	
Iron variables				
Ferritin, pmol/L (log)	93 (82, 105)[c]	88 (72, 105)[c]	98 (82, 114)[c]	NS
Iron, μmol/L	3.7 (2.1, 5.3)[c]	3.6 (1.5, 5.7)[c]	3.9 (1.4, 6.4)[b]	NS
Serum transferrin, μmol/L	−8.3 (−10.2, −6.4)[c]	−6.4 (−9.1, −3.6)[c]	−10.2 (−12.9, −7.5)[c]	0.046
Transferrin saturation, %	2.7 (1.9, 3.4)[c]	2.3 (1.3, 3.3)[c]	3.2 (2.0, 4.4)[c]	NS
Soluble transferrin receptor, mg/L	−48 (−62, −29)[c]	−36 (−58, −2)[a]	−60 (−75, −36)[c]	NS
Ferritin index	−74 (−79, −68)[c]	−91 (−95, −84)[c]	−68 (−75, −60)[c]	<0.001
Red blood cell variables				
Haemoglobin, g/L	0.66 (−0.33, 1.65)	−0.39 (−1.71, 0.95)	1.85 (0.42, 3.31)[a]	0.025
MCV, fL	2.8 (0.9, 4.8)[b]	1.9 (−0.6, 4.5)	4.0 (1.0, 7.0)[b]	NS
MCH, pg/cell	9.7 (4.1, 15.6)[c]	6.5 (−0.6, 14.2)	13.9 (5.2, 23.4)[b]	NS
MCHC, g/L	3.1 (1.3, 5.2)[c]	2.3 (−0.4, 4.9)	4.3 (1.6, 7.2)[b]	NS
Red cell distribution width, %	−14.1 (−22.1, −5.4)[b]	−8.8 (−20.9, 5.1)	−18.4 (−28.4, −7.0)[b]	NS
Anaemia	−27 (−58, 26)	27 (−41, 174)	−58 (−80, −9)[a]	0.047
Inflammation markers				
Fibrinogen, g/L (log)	119 (53, 213)[c]	70 (8, 168)[a]	212 (83, 434)[c]	NS
hs-CRP, nmol/L (log)	14 (7, 22)[c]	12 (3, 23)[a]	17 (6, 28)[b]	NS
Liver and kidney parameters				
eGFR-MDRD <60 mL/min/1.73 m²	40 (−8, 114)	92 (0, 269)[a]	14 (−33, 94)	NS
Alanine aminotransferase, μkat/L (log)	17 (1, 36)[a]	13 (−8, 39)	22 (−2, 51)	NS
Alanine aminotransferase >0.83 μkat/L	37.4 (−8.5, 106.5)	8.9 (−33.0, 77.1)	134.3 (12.4, 388.6)[a]	NS
Dietary variables				
Haeme iron intake, mg/day	40 (9, 82)[b]	25 (−10, 74)	68 (11, 152)[a]	NS
Intake of iron supplements	−48 (−74, 3)		−54 (−77, −6)[a]	
Alcohol intake, g/day	0.43 (−0.00, 0.86)	0.71 (0.25, 1.18)[b]	−1.21 (−2.30, −0.10)[a]	0.002

Values represent the average percent change in hepcidin corresponding to a one unit increase in each row variable, except for variables that were analysed on the (log) scale, for which they represent the average percent change in hepcidin corresponding to a 100% increase in that variable. $p_{Interaction}$ is for tests of effect modification by sex. The column titled "All subjects" provides effect estimates in the sexes combined under adjustment for sex. No significant relationships were found for: Body mass index >30 kg/m², blood donation, hormone replacement therapy (considered only in women), haematocrit, erythrocyte count, hs-CRP >28.6 nmol/L, eGFR-MDRD, γ-glutamyltransferase, iron intake, non-haeme iron intake, alcohol intake >1 g/day. eGFR-MDRD, glomerular filtration rate estimated by the Modification of Diet in Renal Disease formula; hs-CRP, high-sensitivity C-reactive protein; MET-h/week, metabolic equivalent hours per week; MCH, mean cell haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NS, not significant. [a]p<0.05; [b]p<0.01; [c]p<0.001.

with CVD. Of interest, hepcidin serum concentrations in this study were lower than those reported previously for subjects of similar age [17, 32–34], as was the ratio of hepcidin to serum ferritin [17, 35] (Figure 1 and Table 1). While this may be associated with the method of determination [34] or the fact that our subjects had no evidence of acute infection or active ongoing inflammatory disease, the most probable reason is that hepcidin levels are lowest in the morning and increase approximately two-fold during the day [36]. The median concentration we found with blood sampling between 7:00 AM and 9:00 AM (2.27 nM) closely matches that detected previously with sampling at 8:30 AM (2.24 nM) [37].

With respect to the method we used ultra-high pressure liquid chromatography coupled to triple quadrupole mass spectrometry [25], which combines a low limit of detection, a main advantage of immunoassays, with specificity for the bioactive hepcidin-25 isoform, a main advantage of mass spectrometry-based methods [34]. Importantly, we studied subjects that were randomly sampled from the general community, which in combination with the expected low impact of inflammation and

Table 3: Relationships of hepcidin serum concentration with demographic, blood, and dietary variables (95% confidence intervals) under adjustment for age, sex and ferritin.

	All subjects	Men	Women	$p_{Interaction}$
Demographic and lifestyle variables				
Physical activity, MET-h/week	0.24 (0.03, 0.44)[a]	0.20 (−0.06, 0.46)	0.29 (−0.03, 0.61)	NS
Blood donation	8.9 (−12.8, 35.9)	−2.3 (−23.7, 25.1)	67.1 (2.7, 171.9)[a]	NS
Red blood cell variables				
Haemoglobin, g/L	−0.86 (−1.62, −0.10)[a]	−1.32 (−2.33, −0.30)[a]	−0.33 (−1.43, 0.78)	NS
Haematocrit, %	−2.8 (−5.3, −0.2)[a]	−4.0 (−7.4, −0.5)[a]	−1.3 (−5.0, 2.5)	NS
Inflammation markers				
Fibrinogen, g/L (log)	73 (32, 128)[c]	69 (19, 140)[b]	80 (19, 173)[b]	NS
Liver and kidney parameters				
Alanine aminotransferase, μkat/L (log)	−10.9 (−20.9, 0.4)	−19.8 (−31.7, −5.7)[b]	−0.1 (−15.5, 18.2)	NS
Alanine aminotransferase >0.83 μkat/L	−23 (−44, 6)	−40 (−59, −12)[b]	36 (−23, 140)	0.025
γ-Glutamyltransferase, μkat/L (log)	−15 (−22, −8)[c]	−18 (−27, −8)[c]	−13 (−22, −3)[a]	NS
Dietary variables				
Alcohol intake, g/day	−0.38 (−0.71, −0.04)[a]	−0.20 (−0.56, 0.17)	−1.37 (−2.21, −0.52)[b]	0.013
Alcohol intake >1 g/day	−24 (−37, −8)[b]	−24 (−51, 17)	−24 (−38, −5)[a]	NS

Values represent the average percent change in hepcidin corresponding to a one unit increase in each row variable, except for variables that were analysed on the (log) scale, for which they represent the average percent change in hepcidin corresponding to a 100% increase in that variable. $p_{Interaction}$ is for tests of effect modification by sex. The column titled "All subjects" provides effect estimates in the sexes combined under additional adjustment for sex. No significant relationships were found for: Body mass index, body mass index >30 kg/m^2, hormone replacement therapy or postmenopausal state (considered only in women), iron, serum transferrin, transferrin saturation, soluble transferrin receptor, ferritin index, erythrocyte count, MCV, MCH, MCHC, red cell distribution width, anaemia, hs-CRP, hs-CRP >28.6 nmol/L, eGFR-MDRD, eGFR-MDRD <60 mL/min/1.73 m^2, iron intake, haeme iron intake, non-haeme iron intake, intake of iron supplements. eGFR-MDRD, glomerular filtration rate estimated by the Modification of Diet in Renal Disease formula; hs-CRP, high-sensitivity C-reactive protein; MET-h/week, metabolic equivalent hours per week; MCH, mean cell haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NS, not significant; [a]p<0.05; [b]p<0.01; [c]p<0.001.

Table 4: Simultaneous correlates of serum hepcidin concentration.

Variable	Effect (95% CI)	p-Value	t-Value	Partial R^2 (%)
Ferritin, μg/L (log)	87.63 (76.25, 99.75)	<0.001	19.75	32.2
Fibrinogen, g/L (log)	100.50 (51.83, 164.78)	<0.001	4.91	2.0
γ-Glutamyltransferase, μkat/L (log)	−16.30 (−22.56, −9.53)	<0.001	−4.50	1.7
Age, years	−1.18 (−1.95, −0.42)	0.003	−3.02	0.8
Transferrin saturation, %	0.80 (0.16, 1.46)	0.015	2.44	0.5

Values represent the average percent change in hepcidin corresponding to a one unit increase in each row variable, except for variables that were analysed on the (log) scale, for which they represent the average percent change in hepcidin corresponding to a 100% increase in that variable. Partial R^2 represents the change in model R^2 (percent variance explained) upon inclusion of one variable on top of the others.

the high-quality measurement of hepcidin argues that the values we registered are representative for the general population.

Hepcidin was strongly correlated with ferritin, as has been described previously [9, 32, 35, 38–41] (Table 2). In fact, a doubling of hepcidin concentration corresponded roughly to a doubling of ferritin concentration, likely because hepcidin and ferritin are physiologically similarly affected by iron availability [38]. Factors associated with hepcidin independently of iron availability should thus emerge under adjustment for ferritin, such as was performed in Table 3. Safely employing this interpretation requires awareness that hepcidin but not ferritin affects iron availability as a key player in multiple feedback loops [1].

Along this line, men had higher hepcidin concentrations, presumably due to their higher iron stores. However, conditional on ferritin, women had higher hepcidin

Table 5: Associations of serum hepcidin concentration and derived variables with 10-year risk of cardiovascular disease.

Variable	Tertile	All subjects			Women			Men		
		Hazard ratio (95% CI)	p-Value	p_{trend}	Hazard ratio (95% CI)	p-Value	p_{trend}	Hazard ratio (95% CI)	p-Value	p_{trend}
Hepcidin	First	1.00 (Ref.)		0.623	1.00 (Ref.)		0.358	1.00 (Ref.)		0.911
	Second	0.75 (0.45, 1.25)	0.270		0.52 (0.23, 1.21)	0.130		1.01 (0.49, 2.07)	0.983	
	Third	1.00 (0.61, 1.65)	0.992		1.05 (0.52, 2.10)	0.899		1.00 (0.48, 2.07)	0.995	
Hepcidin-Ferritin-Ratio	First	1.00 (Ref.)		0.327	1.00 (Ref.)		0.357	1.00 (Ref.)		0.686
	Second	0.73 (0.45, 1.18)	0.200		0.52 (0.24, 1.12)	0.096		0.90 (0.49, 1.68)	0.747	
	Third	0.69 (0.40, 1.18)	0.178		0.58 (0.28, 1.22)	0.152		0.80 (0.37, 1.75)	0.583	
Hepcidin-TFS-Ratio	First	1.00 (Ref.)		0.689	1.00 (Ref.)		0.410	1.00 (Ref.)		0.948
	Second	0.63 (0.37, 1.07)	0.085		0.56 (0.26, 1.23)	0.150		0.73 (0.35, 1.52)	0.398	
	Third	0.95 (0.58, 1.56)	0.844		0.77 (0.37, 1.57)	0.470		1.11 (0.55, 2.25)	0.767	

CI, confidence interval; Ref., reference group; TFS, transferrin saturation. There occurred 91 incident CVD events in 675 subjects. Corresponding numbers by sex are 50 events in 325 men, and 41 events in 350 women. Analyses were adjusted for age, sex, C-reactive protein, anaemia, and the Framingham risk score variables diabetes mellitus, current smoking, systolic blood pressure, and total and high-density lipoprotein cholesterol.

concentrations, which was also reflected in a higher hepcidin-to-ferritin ratio in women and has been described before [17, 35]. This observation may be linked to the effect of different hormones on hepcidin regulation [3, 4, 7], as few women in this study were premenopausal (4.3%).

All associations between hepcidin levels and iron parameters were highly significant without but non-significant with adjustment for ferritin (Table 3). Given that hepcidin is regulated by circulating iron levels independently of tissue iron stores [1, 2], it is unexpected that we, as others before [32, 35], found no respective independent association. This may reflect that metabolically available iron is rapidly incorporated into ferritin and a steady state between circulating ferritin, iron, and hepcidin develops.

The relationships of hepcidin with red blood cell indices were strongly affected by iron status, and iron deficiency was associated with low hepcidin levels and microcytic anaemia (Table 2). Following adjustment for ferritin, a negative association of haemoglobin with hepcidin became evident, which tended to be more pronounced in men (Table 3). This picture is more consistent with anaemia of chronic disease or anaemia of inflammation, a normocytic anaemia in which hepcidin levels and ferritin are normal to high [42]. Naturally, these are putative aetiologies; anaemia in the elderly is often of multifactorial aetiology [43]. Given the low prevalence of definite anaemia as defined by haemoglobin cut-off (<120 g/L in women, <130 g/L in men) in our sample (Table 1), subjects with low-normal haemoglobin levels may have contributed significantly to these associations, which would be in line with the high prevalence of predominantly mild anaemia in the elderly [43].

Figure 2 provides a more detailed description of the aetiologies of definite anaemia in this cohort of apparently healthy subjects. In comparison to a study conducted in 85 year olds [33], fewer subjects in our cohort had anaemia of chronic disease, anaemia of chronic kidney disease, or anaemia of multiple aetiologies.

C-reactive protein (CRP) and fibrinogen were positively associated with hepcidin under adjustment for age; all three proteins are induced by interleukin-6 (IL-6) [44]. However, the association of hepcidin with CRP lost significance when adjusting for ferritin and for body mass index, possibly because ferritin is also an acute phase protein, or because low-grade inflammation was rare in this general community cohort. In contrast, the relationship of hepcidin with fibrinogen remained significant under the same adjustment, and also under additional adjustment for CRP. This links hepcidin to fibrinogen and coagulation which has been first explored upon the observation that heparins can modify hepcidin expression in vitro and in vivo [45].

Interestingly, hepcidin was negatively associated with γ-GT levels under adjustment for ferritin but not without such adjustment, possibly due to down-regulation of hepcidin by alcohol [46]. Chronic sizeable alcohol consumption, as was prevalent in study subjects, is expected to decrease hepcidin [46] and favour iron accumulation [47] while increasing γ-GT. Hepcidin levels in those with high alcohol intakes might therefore have been upregulated through increased iron stores and down-regulated through ongoing alcohol consumption itself, resulting in a null association with γ-GT when not conditioning on iron stores.

Similar considerations may explain the significant sex differences in the relationship between average long-term alcohol consumption and hepcidin levels. In men, this relationship was consistent with hepcidin reflecting iron retention, as is plausible given their substantial alcohol intakes. Women consumed much less alcohol such that the direct effect of alcohol on hepcidin expression may have had relatively greater weight.

Dietary haeme but not non-haeme iron intake was positively associated with hepcidin only in women. This is coherent given that iron absorption adapts to iron requirements in healthy individuals, women in our sample had lower iron stores than did men, and haeme iron is more efficiently absorbed than non-haeme iron [1, 2, 43].

Table 4 shows the selected independent predictors of hepcidin serum concentration. It reinforces the overwhelming dependence of hepcidin on ferritin levels, and shows that conditional on its most important other modulators, hepcidin levels decline with age and are dependent on circulating iron levels which are lower with more advanced age [1]. This also confirms experimental data that iron availability dominates inflammation in regulating hepcidin expression [1, 48].

We found no significant associations of hepcidin, of hepcidin-to-ferritin ratio, or of hepcidin-to-transferrin saturation ratio with carotid atherosclerosis or with CVD (Online Table 1 and Table 5). This contrasts to the results of a recent report which linked increased hepcidin or hepcidin-to-ferritin ratio to the presence of carotid plaque in healthy postmenopausal women [17]. Differences in study design may account for this discrepancy: Galesloot and colleagues [17] correlated hepcidin measurements to the presence of plaque 3–5 years later, while we assessed hepcidin and atherosclerosis at the same time point. Their sample may have been subject to more pronounced selection effects, as of 22,451 subjects initially randomly selected, only 766 were included, while in this study, of 702 subjects participating in 2000, 694 were included. Importantly, hepcidin levels were substantially higher in their study, and the levels they found associated with excess plaque occurred in only 2% of our female participants, leaving the possibility that only very high hepcidin levels are related to plaque. In that case it will be interesting to identify the source of such high levels.

Our results are in line with previous findings in *Apoe*$^{-/-}$ mice that hepatic hepcidin expression was unaffected by the progression of atherosclerosis [14], and that atherosclerotic plaque size was not increased with elevated macrophage iron [14], findings that question the notion that hepcidin exerts proatherogenic effects by promoting macrophage iron retention [10].

There are other epidemiologic studies reporting positive associations of hepcidin with vascular endpoints, including arterial stiffness [19] and cardiovascular events [18] in patients on maintenance haemodialysis. These findings in diseased subjects are of interest but may not necessarily apply to healthy subjects.

Strengths of our study include that its participants are well characterised and near-ideally representative for the general population, that blood was sampled at uniform time points and in the absence of acute infection in all subjects, ruling out major influences of inflammation [1] and of diurnal variations [36] on hepcidin levels. As this is a population-based study investigating a representative random sample of healthy adults, significant selection bias was unlikely. Among its weaknesses is that, while a sufficient number of subjects with atherosclerosis were included, there were relatively few subjects that experienced CVD events in each sex group, and that we had no data on insulin resistance, which may be an important determinant of hepcidin concentrations [49]. Another potential weakness is that we abstained from correcting for multiple testing; we performed many at times highly correlated tests, and in this setting correcting for multiplicity while maintaining acceptable power is challenging.

In summary, in this study involving elderly subjects from a general population, γ-GT and fibrinogen were the most significant independent correlates of hepcidin only surpassed by ferritin, and hepcidin was not associated with prevalent carotid atherosclerosis or with incident cardiovascular disease.

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